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1. Your reference

IBST Ø91

2. Patent application number
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3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

CRANFIELD UNIVERSITY, CRANFIELD, BEDFORDSHIRE, MK43 0AL, UK AND DIACLONE, PO Box 1985, 1 BOULEVARD FLEMING, 25020 CEDEX, BESANCON, FRANCE

Patents ADP number *(if you know it)*

0645848007

If the applicant is a corporate body, give the country/state of its incorporation

0866598001

4. Title of the invention

IMMUNOCHEMICAL METHODS AND REAGENTS FOR MEASUREMENT OF FUEL OXYGENATES

5. Name of your agent *(if you have one)*

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Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*
—		

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day / month / year)*
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8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*

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Signature L.J. Tidwell Date 30-06-03

12. Name and daytime telephone number of person to contact in the United Kingdom

LINDA TIDWELL
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Immunochemical methods and reagents for measurement of fuel oxygenates

Field of the Invention

The present invention relates to immunochemical assays and associated immunoreagent production methods for determination of members of the *tert*-butyl group of fuel oxygenates and associated compounds. The approach, comprising an immunochemical-based assay and associated reagents may be used in either the laboratory of the field for the rapid and routine diagnosis of samples suspected of containing said compounds thereby allowing rapid analytical and diagnostic information to be obtained.

Background to the invention

Human endeavour is hugely dependent upon the consumption of fuels for power generation purposes. For example, road transport is an integral feature of today's society, essential for industry, commerce and recreational activities. Global demand for petroleum necessitates usage of large fuel storage tanks and underground oil pipelines, which present the potential for significant environmental contamination from persistent and hazardous petroleum hydrocarbons. Particularly with underground systems, leaks can remain undetected and can be transported through soils and water systems via natural dispersion mechanisms.

From the early 1970's, increasing concern about public health and environmental impacts from exhaust emissions has led to changes in the formulations of motor fuels. Revised European and US regulations have been targeted to reduce emissions from motor vehicles and improve air quality. Oxygenated compounds have been incorporated into fuels to aid combustion and reduce exhaust emissions responsible for air pollution. These provided a replacement for benzene and have aided fuel

producers in reaching octane levels required by new fuel standards.[1] The choice of oxygenate varies between countries and US states, influenced by the availability of raw materials, individual economies and political status.

Currently, the most commonly used fuel oxygenates are methyl *tert*-butyl ether (MTBE) and ethanol. Other oxygenates in use include ethyl *tert*-butyl ether (ETBE), methyl *tert*-amyl ether (TAME), isopropyl ether, and *tert*-butyl alcohol (TBA). These compounds have been primarily used to achieve mandatory fuel standards throughout the US and Europe. Whilst petroleum may comprise of >120 different compounds, leaded, unleaded and premium grade fuels may contain >15% oxygenates by volume.

MTBE has since become one of the most significant environmental pollutants in recent years. Sampling results from the US Geological Survey's (USGS) National Air and Water Quality Assessment (NAWQA) Programme during 1990-1998 found high incidences of MTBE contamination in both confined and unconfined ground water aquifers and in drinking water.[2] Approximately 60% of US drinking water is extracted from surface water systems. Whilst the potential toxicity is still debated, MTBE has an extremely low taste threshold and low-level contamination has led to losses of drinking water supplies. 71% of the water supply of Santa Monica, California, has been tainted with MTBE, requiring the importation of water supplies from outside of the city limits at an annual cost of $3.5 million per annum.[3] MTBE has been

[1] Environment Agency (2001) National groundwater and Contaminated Land Centre Annual Report 1999/2000.

[2] Moran, M.J. *et al.* (1999) Relations between the detection of MTBE in surface and groundwater and its content in gasoline. US Geological Survey Report, 1999.

[3] Environment Agency. The fuel additive MTBE – a groundwater protection issue? National Groundwater and Contaminated Land Centre.

increasingly identified as the primary threat to European ground water reservoirs, which supply 60-70% of all European drinking water.

The main source of environmental oxygenate contamination is from oxygenate-blended petroleum, through leaking underground storage tanks (LUSTs), transfer spillage, petroleum facilities and/or accidental spills.[4] Poor management of stored fuel and subsequent leakages have resulted in major, global environmental contamination from petroleum and related components, typically BTEX compounds (benzene, toluene, ethylbenzene and *m*-, *o*-, *p*-xylenes), low weight alkanes, such as *n*-pentane, *n*-hexane, and fuel oxygenates. MTBE has particular environmental significance due to its high solubility in water, with numerous incidences of ground water and/or drinking water contamination. Surface water systems may become contaminated through ground water, atmospheric deposition, storm water run-off and direct releases by industrial and recreational activities.[5]

State of the art

Traditionally, environmental analysis of petroleum and related compounds are performed using laboratory-based methods such as gas or liquid chromatography allied to suitable detection methodologies. The two most widely used analytical methods for detection of fuel oxygenates are EPA Method #8260 (Volatile organic compounds by Gas Chromatography/Mass Spectroscopy (GS/MS) and Method #8015 (Non-halogenated organics using Gas Chromatography/Flame Ionisation Detector (GC/FID). Additionally, there are problems with sample collection methods and sample preservation for these laboratory-based techniques. Whilst a wide range of

[4] Environment Agency (2001). Fuelling Stations Construction and Operation. Environmental Agency Pollution Prevention Guidelines PPG7.

[5] Bradley, P.M. *et al.* (1999). Aerobic mineralization of MTBE and *tert*-butyl alcohol by stream bed microorganisms. Environmental Science and Technology, 33, pp. 1877-1879.

analytical techniques are available, these remain primarily laboratory-based. Due to escalating reports of petroleum and specifically MTBE contamination across the US and Europe, there remains an urgent need for simple and accurate diagnostic tools for *in-situ* analysis and continuous monitoring applications.

Immunoassays

The specific, sensitive and low-cost *decentralised* determination of a number of different types of common environmental contaminant has been achieved through the use of immunoassays. Immunoassays allow the detection and measurement of target compounds using specific binding characteristics of antibodies and can be found in a wide variety of formats and are increasingly being developed and employed for environmental monitoring purposes. These have acquired wide acceptance in the USA, with the US EPA recognising and releasing official ELISA methods for the determination of certain compounds, e.g. certain pesticides, PAHs and PCBs. 'Rapid' immunoassay test kits are commercially available for a wide range of analytes, which provide a relatively inexpensive, rapid (<2 h), sensitive screening method for analyte detection, commonly in aqueous and soil matrices. Castillo *et al.*[6] have evaluated the immunoassay test kits for the accurate and sensitive determination of pentachlorophenol, carcinogenic PAHs and BTEX compounds within industrial effluents.

Fundamental principles of immunoassays

The fundamental principle of immunoassays (IAs) is that they utilise biologically generated immunoglobulin proteins – antibodies (Ab) - which react with specific target compounds – namely the target analyte, commonly referred to as the 'antigen'

[6] Castillo, M. *et al.* (1998). Environmental Science and Technology, Vol. 32(14), pp. 2180-2184.

(Ag) to form antibody-antigen complexes (Ab-Ag). All IAs are based on the selectivity and sensitivity of this Ab-Ag reaction. Due to the wide range of variations in assay design, there is no universal, consistent classification system, or terminology. Each immunoassay will need to be specifically developed to the target of interest, i.e. the antigen. The Ab is the key reagent and these generally determine the assay's characteristics. Ab-Ag binding arises from structural complimentarity between the 2 molecules, stabilised by binding through a combination of Van der Waals forces, electrostatic interactions, hydrogen bonding and hydrophobic interactions.

Antibody production

The initial stimulation of Immunoglobulin G (IgG) antibodies is achieved through injecting animals, commonly mice, rabbits or sheep, with the target immunogen (antigen). The *in vivo* administration of an immunogen stimulates B-lymphocyte cells to produce and secrete antibodies into the blood stream that are capable of binding to, and with the help of other factors in the immune response, destroying the invading blood-borne foreign body. Since each stimulated B-lymphocyte cell will produce a unique antibody 'clone' exhibiting a specific binding reaction, and hence affinity, to the target analyte, a 'polyclonal' mixture of antibodies derived from all of the individual Ab secreting cells, is elaborated within the serum. This polyclonal antiserum can be utilised at this stage, although the IgG fraction will contain many antibodies of differing specificities, many of which may be irrelevant for IA purposes.[7] A purification step is generally necessary to increase performance and limit the possibility of activity from those irrelevant fractions.

[7] Edwards, R. (1996) (Ed.) Immunoassays. Essential data Series. John Wiley and Sons, Chichester, UK.

A problem with polyclonal Ab preparations are that there are significant batch-to-batch variations in the quality of the antiserum and the source of the preparation will cease on death of the animal host. This is a particular problem in immunoassay manufacture when product consistency is paramount to maintaining a valid analytical/diagnostic tool. This issue has been overcome through the development of monoclonal antibody production methods, which exploit the use of neoplastic multiple myeloma 'tumour' cells – essentially Ab secreting cells that undergo uncontrolled and rapid cell division. Individual Ab secreting cells are isolated from the animal host, and fused with these myeloma cells to form hybridoma cells that, with careful cultivation, act as effectively immortal cell lines for the production of individual cloned (monoclonal) Ab preparations. Generally, vast numbers of hybridoma cells are generated and then screened for Ab-Ag binding efficacy. The high purity, homogeneity and cloning ability of MAbs enables easier purification and subsequent labelling of these highly specific antibodies. Although costs and practical investments are initially high, these are now the preferred and established practice for IA design.

A significant issue arises in that most organic pollutants are of insufficient molecular weight to engender an immunogenic response. Molecules with a molecular weight of <3000 are not immunogenic and those <5000 may be too weak to induce an adequate immunogenic response. In these cases, the molecules must be conjugated to a much larger carrier protein in order to provoke an immune response. Both MTBE and ETBE have molecular weights of 88 and 102 respectively and are termed 'haptens' (i.e. MW around 100 or less) and thus need to be conjugated in order to stimulate host antibody production. Only those antibodies binding specifically to the haptenic determinant, as opposed to the carrier protein will be of diagnostic use. Theoretically,

various immunogenic carriers can be used, the most common being bovine serum albumin (BSA), human serum albumin (HSA), rabbit thyroglobin and keyhole limpet haemacyanin (KLH).

Immunoassay Design

There are many possible immunoassay formats reported in the literature that exploit the fundamental principle of quantifying the extent of Ab-Ag binding. The method most used for field-based determinative purposes is termed the indirect competitive assay format. The method is indirect in that one of the immunoreagents must be labelled in order to visualise the extent of Ab-Ag binding and hence quantify the amount of target analyte in the sample solution. The choice of label remains largely dependent on the specific characteristics and expected concentrations of the target analyte.

Suitable labels include enzymes, fluorochromes and radioisotopes, the latter being less widely used in recent years. Enzymes can also be selected to convert non-fluorescent substrates to fluorescent products, e.g. alkaline phosphatase. Photoluminescent compounds in immunoassays (FIAs) can provide even greater sensitivity that colorimetric substrates. Fluoroscein isothiocyanate (FITC) is usually the label of choice for immunofluorescent IAs, as coupling procedures are straightforward works with almost all antibodies. Additional labels commonly used include tetramethylrhodamine isothiocyanate (TRITC), Texas Red (TR) or phycoerythrin (PE).

Enzyme Linked Immunoassays (ELISA)

Enzyme linked immunosorbent assays (ELISAs) are based on the combination of selective antibodies with sensitive enzymes that react with a substrate to produce a

detectable colour change, e.g. commonly horseradish peroxidase (HRP), or alkaline phosphatase. Specific enzymes are incorporated to link to target contaminants and through enzymatic actions on the colouring agent (chromogen), enabling both qualitative and quantitative analysis through the catalytic capability of the enzymes. Many regulated contaminants, such as pesticides, polyaromatic hydrocarbons (PAHs) and other organic pollutants can now be detected on-site using available test kits utilising enzyme linked immunoassay technology. A number of solid supports can be used as a means of separation in an ELISA system. Solid support within ELISA systems can be the traditional microtitre plate systems, coated tubes or using covalently bound antibodies to magnetic or latex particles. Due to their relative simplicity, flexibility, speed and cost, IAs are now generally considered an effective and suitable form of analysis, suitable for both laboratory and field diagnostics. Such IA systems are capable of detecting very low levels of contamination, in some cases as low as the ng/L or ppb level.[8]

For petroleum contaminants, a number of IA based field kits are commercially available for on-site analysis, e.g. the Strategic Diagnostics Incorporated RaPID Assay Petroleum Fuel Kits. These field kits utilise Abs bound onto microscopic particles, for which results are interpreted using a portable microprocessor controlled spectrophotometer or photometer. Detection levels can be at µg/L (ppm) or ng/L (ppb) concentrations, depending on the analyte in question and product used. Reportedly, results can be obtained in less than two hours.[8] However, whilst the IA aspects can be accomplished in some cases <60 minutes. A further extraction procedures are necessary for soil analysis, which increase the complexity and time scale of analysis.

Summary of the invention

There is extreme current interest in MTBE and related fuel oxygenates -ETBE, TAME, TBA etc. - hereafter referred to as the *tert*-butyl oxygenate family, and their environmental impact. Despite this fact there is no prior art concerning the development of immunochemical methods for determination of such compounds. It is worthy of note that commercially available test kits to the other major classes of petroleum species, such as the BTEX compounds, TPHs (total petroleum hydrocarbons) and PAHs (polynuclear aromatic hydrocarbons) do currently exist.

This invention therefore pertains to the processes, technologies and associated knowledge associated with the generation of immunoreagents and the development of immunoassays for the antibody-based determination of members of the *tert*-butyl oxygenate family. The benefits of such an approach lie in the simplicity of the assay procedure and amenability of said procedure to decentralised usage. The method offers the following benefits over current MTBE determination methods:

- The simplicity of the procedure and requirement for simple instrumentation (optical reader, electrochemical monitoring device or such other signal interrogation device) renders the method amenable to decentralised operation.

- High sample throughput: achieved through the ability to assay a large number of samples simultaneously.

[8] http://www.sdix.com

- A high degree of assay specificity and sensitivity due to the binding complimentarity and binding affinity between the antibody and target analyte(s).

- Assay rapidity, typically in the region of 1-2h.

- *In situ* sample determination removes the requirement for transportation of samples to a centralised facility and the concomitant degradation of sample during transportation and storage.

- Despite the costs associated with antibody preparation, particularly monoclonal antibody preparation, the small amounts of Ab material and other reagents required for each assay measurement results in low assay costs relative to the costs associated with running complex instrumentation at dedicated centralised analytical facilities.

- Operator costs are reduced due to the speed/throughput of the assay and the lower levels of training required relative to their laboratory counterpart.

In order to generate antibodies with binding specificities directed towards the *tert*-butyl oxygenate family, account must be made of the extreme low molecular weight of these compounds. Research in our laboratories has focussed on producing *tert*-butyl oxygenate-protein conjugates capable of engendering an immunological response in a host animal species.

One critical aspect of this invention relates to the synthesis and utilisation of *tert*-butyl oxygenate analogues for immunogen production. These analogues may be polymers (dimers, trimers, polymers etc.), consisting of polymerisation of two or more *tert*-butyl oxygenate monomers, to form extended repeated *tert*-butyl oxygenate polymer chains, or alternatively, individual molecules exhibiting structural and functional characteristics of the *tert*-butyl ether target compound in association with a second entity that acts as a spacer between the tert-butyl ether functionality and associated carrier compound. Additionally, these compounds will also contain a functionality located away from the tert-butyl ether moiety that can be used for carrier compound conjugation purposes, such as conjugation to suitable carrier proteins. Such entities, henceforth referred to as [*tert*-butyl oxygenate]$_n$-carrier protein conjugates (i.e. *tert*-butyl ether polymer structures and *tert*-butyl ether-spacer compounds) were found to elicit the desired immunological response in host animals, with recovery of antibody with specificity and high binding affinity for both the *tert*-butyl oxygenate polymer and original monomer compound. Simple conjugation of members of the *tert*-butyl oxygenate family to standard carrier proteins yielded antibodies with significantly weaker antigen binding affinity.

This invention further relates to the use of methodologies whereby the specific binding capabilities of these antibodies, produced using either the *tert*-butyl oxygenate polymer-carrier protein or *tert*-butyl oxygenate monomer-carrier protein immunogen route, are exploited for the creation of immunodiagnostic methods for the determination of members of the *tert*-butyl oxygenate family. The immunoassay format may vary in nature but relates to the use of immunochemical assay formats and specific assay labels that are able to visualise the extent of Ab-Ag binding and hence lead to the determination of the *tert*-butyl oxygenate family members.

The assay format may be competitive or non-competitive in operation and may include alternative embodiments of either approach, such as the use of particulates, such as latex beads, magnetic beads and the well described lateral flow assay format. The assay label may be linked directly to one or more of the immunoassay reagents, such as antibody or antigen analogue, or may be introduced by alternative means, such as via the well-known streptavidin-biotin binding complex, or through conjugation of the label to a second antibody or binding component with binding specificity directed towards structures on the primary anti-*tert*-butyl oxygenate antibody. The label may be, but is not limited to an enzyme, chromophore, fluorophore or other optically active agent, chemically active agent, electrochemically active agent or other such suitable compound in which the specific properties of the label can be used to visualise the Ab-Ag binding process. In the case of enzyme labels, the consumption of active substrate and/or the generation of active product or other enzyme mediated effect may be used to produce the assay response. Selection of the transduction process which will be dependent upon the selected assay label may include, but not be limited to optical, electrochemical or other appropriate method. Integration of the immunoassay with appropriate transduction methodologies to produce dedicated sensing tools is a further clear embodiment of the invention described herein.

Whilst it is evident that the invention is suitable for decentralised determination of members of the *tert*-butyl oxygenate family using the indirect immunochemical assay format, it is evident that the methodologies herein described may be equally applied to direct immunochemical assay methods. Direct methods are able to visualise the Ab-Ag binding process without the aid of associated assay labels by use of appropriate transduction methodologies. Such methodologies would include, but would not be

limited to: surface plasmon resonance (SPR) devices, evanescent wave devices, quartz crystalline microbalance (QCM) devices, surface/bulk acoustic wave devices, field–effect transistors or any other such methodology that may be considered direct in operation.

Specific example

A specific embodiment of the invention is now given by way of the following example.

An indirect competitive immunoassay with specificity towards MTBE has been constructed and tested with representative samples. The assay is centred on the competition between microtitre well wall immobilised MTBE-spacer-BSA polymer (7-methoxy-3,7-dimethyloctanal-BSA - the 'coating antigen'), free MTBE in the sample and free anti-MTBE/MTBE-spacer antibody.

Anti-MTBE/MTBE-spacer antibody was first generated by conjugating 7-methoxy-3,7-dimethyloctanal to BSA carrier protein. The resultant conjugate was injected into a mouse host and antibody synthesising cells harvested at the appropriate time. The antibody synthesising cells were fused with myeloma cells to form hybridoma cells which were cultivated and the antibody specificity of each hybridoma assessed with respect to binding to MTBE and 7-methoxy-3,7-dimethyloctanal. Those hybridomas expressing antibody of the required specificity were propagated and used as a source of anti-MTBE/7-methoxy-3,7-dimethyloctanal antibody, for immunoassay applications.

A competitive immunoassay is described in this particular embodiment. Free MTBE antigen present in the sample was allowed to compete with the immobilised coating

antigen for anti-MTBE/7-methoxy-3,7-dimethyloctanal antibody. The quantitative nature of the assay is evident from the fact that the final signal response generated by the assay is dependent upon free MTBE-Ab binding. In essence, the lower the concentration of free MTBE in the sample solution, the greater the extent of antibody binding to the coating antigen. On washing the microtitre wells, free MTBE-antibody complexes are displaced from the well. An antibody-specific enzyme tracer is then added to the system. Following the binding and washing steps, residual enzyme activity, which is inversely proportional to the original free MTBE concentration, is then determined by adding enzyme substrate and recording the optical density of the coloured enzymic product after a suitable period of colour development. Low concentrations of MTBE in the sample gave an inversely proportional large signal output, whilst high concentrations of free MTBE in solution resulted in a reduced assay signal.

Microtitre well preparation

Wells were prepared by adding 100µl of coating antigen (500ng/ml) and then incubated at 4°C for 24 hours. Plates were then aspirated and washed with wash solution (0.01% v/v Tween 20 in Reverse Osmosis (RO) water) at 300µl/well. After each wash step, plates were inverted and blotted against clean paper towelling. Unless otherwise stated, plates were blocked using 5% v/v BSA blocker buffer and incubated at room temperature (RT) for 2 hours. Plates were then aspirated and two wash steps performed. Dried plates were then sealed and stored with desiccant at 4-8°C prior to use.

ELISA immunoassay procedure

A 100µl volume of MTBE-containing sample was added to each microplate well followed by 100µl of biotinylated anti-MTBE/7-methoxy-3,7-dimethyloctanal Ab.

The plates were then sealed and incubated for 1 hour at RT. The aspiration and wash step, as described for plate preparation, was repeated ×4. Streptavidin-HRP (100µl per well) was added, the plates sealed and incubated at RT for a further 30 minutes. Aspiration and 4 further wash steps were made. To each well, 100µl of the HRP enzyme substrate TMB (3,3',5,5' tetramethyl benzidine) was added. The plates were then covered and incubated for 10 minutes. At the end of the colour development time, the colour reaction was stopped by the addition of 100µl 2M H_2SO_4 and the OD_{450} immediately measured. Any necessary dilutions were made with RO water.

Results

Optimal concentrations of both Ag and Ab (biotinylated and native) were determined by checkerboard assay (Figure 1). The optimal concentrations of coating antigen conjugate and biotinylated Ab were all found to be 500ng/ml.

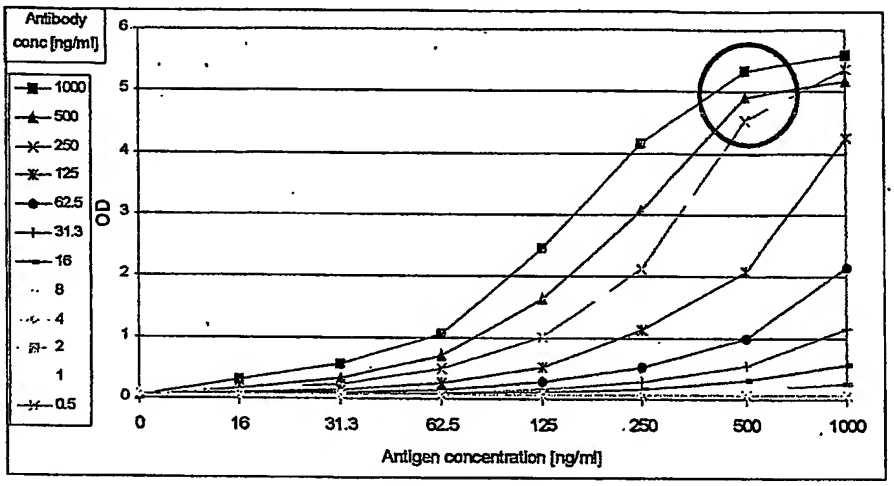

Figure 1. Checkerboard assay for the determination of optimal coating Ag and primary Ab concentrations. The highest signal was achieved using 500ng/ml coating antigen and 500µg/ml anti-MTBE/7-methoxy-3,7-dimethyloctanal antibody.

A 500 ppm MTBE stock solution was prepared in 100 mM phosphate buffered saline (PBS, pH 7.4) and serially diluted (1/10) in 100 mM PBS to a final concentration of 0.5 ppb. A competitive ELISA was then performed. Figure 2 shows a reproducible sigmoidal curve of assay response (OD_{450}) as a function of increasing free MTBE concentration. As the ELISA is a competitive assay, the OD_{450} is inversely proportional to increasing concentrations of free MTBE. The assay was repeated on different days and with fresh solutions of all reagents. Results are shown in Figure 3.

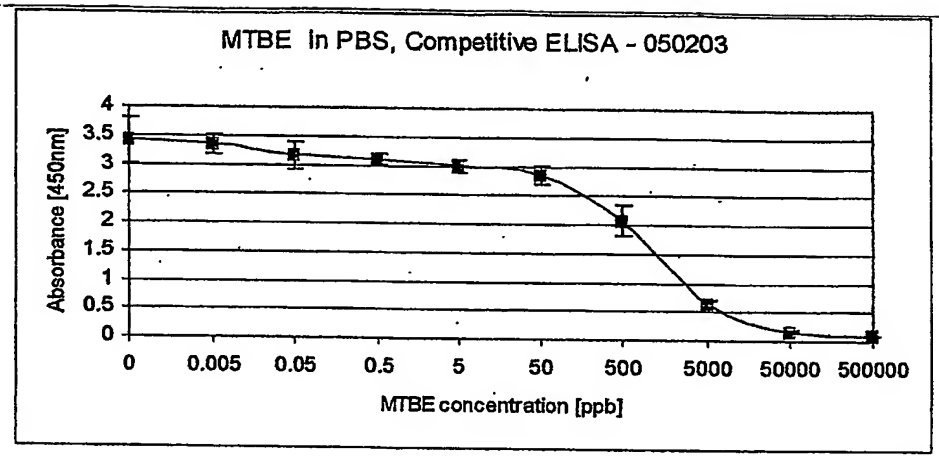

Figure 2. Standard ELISA result comparison with spiked MTBE samples in PBS. The dynamic range of the assay was found to be 50 – 5000 ppb for free MTBE.

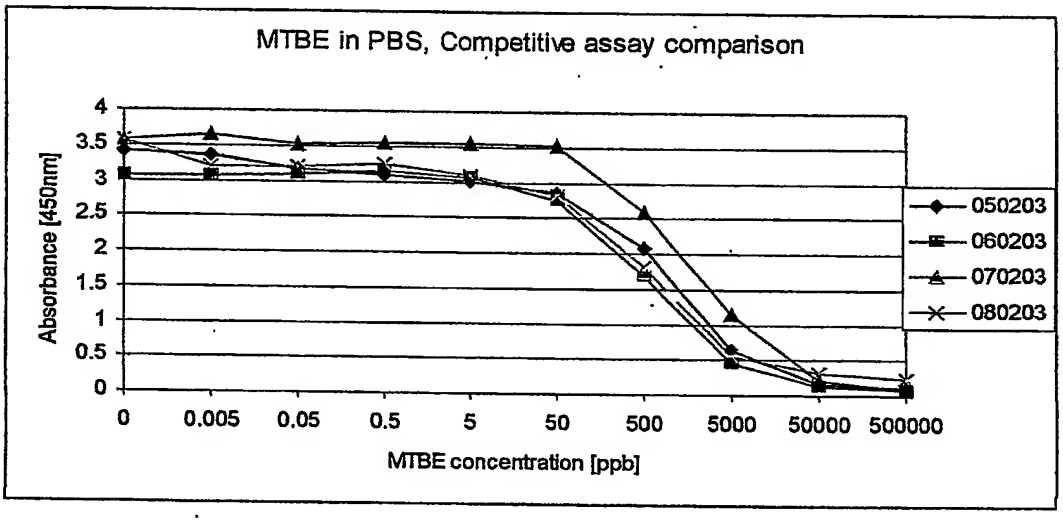

Figure 3. Standard ELISA result comparison with spiked MTBE samples in PBS. The dynamic range of the assay was found to be 50 – 5000 ppb for free MTBE.. Plates 050203 and 060203 were blocked with 5 % v/v BSA blocking buffer. Plates 070203 and 080203 were blocked with 1% BSA blocking buffer.